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Evaluation of Nested PCR in Diagnosis of Typhoid Fever

Pradyot Prakash,[1]* Om Prakash Mishra,[2] Alok Kumar Singh,[3]
Anil Kumar Gulati,[1] and Gopal Nath[1]

Department of Microbiology[1] and Pediatrics,[2] Institute of Medical Sciences, and Department of Botany,[3] Faculty of Science, Banaras Hindu University, Varanasi, India

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In this study, nested PCR using *H1-d* primers, which is specific for *Salmonella enterica* serovar Typhi, was compared to blood culture and the single-tube Widal test. Results indicate that nested PCR can be used as a gold standard to determine the cutoff titer of the Widal test for diagnosis of typhoid fever.

Typhoid fever is a systemic illness caused by *Salmonella enterica* serotype Typhi, and it is endemic in developing countries. Although blood culture is considered the gold standard for the diagnosis of typhoid fever, its results are often jeopardized due to prior inadequate doses of antimicrobials (10). The specific gene sequence of the bacterium, which can be amplified and detected specifically and rapidly by nested PCR (4, 13), is another important target, having the sensitivity of detecting even one bacterium in a given sample within a few hours (7). Moreover, detecting antibodies by the Widal test can also yield a diagnosis of typhoid fever. However, this test carries many shortcomings, as the sensitivity, specificity, and predictive values differ in different geographic areas (9, 11, 12, 15). For better utilization of this test, an appropriate cutoff titer must be determined for a particular geographic area in relation to the ideal gold standard test. Such a cutoff titer cannot be proposed until a proper gold standard test is determined.

This study was conducted in the University Hospital, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India. A total of 63 clinically suspected cases of typhoid fever, in the age group of 1 to 12 years, were included based on presentation with continuous high-grade fever, toxic appearance, splenomegaly (<3 cm), and constitutional symptoms. Patients of both sexes were included and were comprised of those attending the outpatient department and also hospitalized cases, irrespective of their prior antityphoid treatment. The control group consisted of 25 healthy children in the same age group without febrile illness the preceding 6 months. Appropriate amounts of blood were collected from each child for Widal tests, 2 to 8 ml of blood in brain heart infusion broth with sodium polyanethol sulfonate was collected for culture isolation, and 3 ml of blood in a container also holding citrate phosphate buffer (pH 7) was collected for the PCR-based study.

DNA extraction was done from whole-blood samples by using the phenol-chloroform method (13), with few modifications. Nested PCR was performed as described by Song et al. (13) and was modified according to Frankel (4). The reaction mixture for the first-round PCR contained 2.5 μl of 10× PCR buffer (MBI Fermentas, Hanover, Md.), 1.1 μl of (1.5 mM) $MgCl_2$ (MBI Fermentas), 11 pmol of each primer ST1 and ST2 (QIAGEN Operon, Cologne, Germany), 1 μl of deoxynucleoside triphosphate mix (MBI Fermentas), 1 U of *Taq* DNA polymerase (MBI Fermentas), 10 μl of DNA template, and water to a final volume of 25 μl. The first-round amplification was carried out in a thermocycler (Biometra, Goettingen, Germany) under the following conditions: 40 cycles for 1 min and denaturation at 94°C, 1 min 15 s annealing at 57°C, and 1 min elongation at 72°C, with a final elongation step extended to 7 min.

The nested PCR master mix was the same as that of the first-round PCR, except it contained 21 pmol of each primer ST3 and ST4 and 4 μl of DNA template (1:6-diluted product of the primary cycle). Thermal cycling was carried out as described for first-round PCR, except that the annealing temperature was set to 63°C. To separate amplified products, 5 μl of solution was electrophoresed on a 1.5% agarose gel in TBE (Tris-borate-EDTA) buffer at 80 V for 1 h. The gels were stained with ethidium bromide, and the bands were visualized under UV light.

For isolation of the bacterium, the blood culture bottles (HiMedia, Mumbai, India) were incubated at 37°C for up to 7 days, with subcultures taken on alternate days. The isolates were identified by following standard methods (2). The Widal test was performed by using a colored antigen kit (Span Diagnostics, Surat, India).

For statistical analysis, positive predictive value (PV^+), negative predictive value (PV^-), likelihood ratio for a positive test result (LR^+), and likelihood ratio for a negative test result (LR^-) were calculated (5). A z test was applied to determine the significance between two proportions (3).

None of the afebrile healthy control subjects was positive for the bacterium by PCR and blood culture. However, one (4%) child of this group was observed to have an antibody titer against somatic (TO) antigen of 160. Of the 63 suspected cases, nested PCR was positive in 53 (84.1%) cases, the Widal test was positive in 41 (65.0%) cases at the cutoff titer 160 for O and/or H agglutinins, and positivity by blood culture was observed in 17 (26.9%) cases. The positivity of PCR was significantly higher ($P < 0.01$) than that of blood culture and Widal.

When the two most specific tests were evaluated in the suspected cases of typhoid fever that were positive by any of the three tests employed ($n = 57$), PCR was found to have sensitivity of 92.8% while blood culture had only 29.8% sensitivity. Few reports on the application of PCR in the diagnosis

* Corresponding author. Mailing address: Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India. Phone: 91-9415201684. E-mail: pradyot_micro@yahoo.co.in.

TABLE 1. Statistical evaluation of blood culture and a single Widal test (n = 53)

Test	Sensitivity (%)	Specificity (%)	PV^+	PV^-	LR^+	LR^-
Blood culture	32.0	100.0	100.0	21.7	∞	0.67
Widal test[a]	79.2	30	85.7	21.4	1.13	0.69
Widal test[b]	69.8	60	90.2	27.2	1.74	0.50

[a] Cutoff titer of TO, ≥1:80; of TH, ≥1:160.
[b] Cutoff titer of TO, ≥1:160; of TH, ≥1:160.

of typhoid fever from areas where it is endemic have shown lower sensitivity than that observed in the present study (1, 6, 8). The reasons for the better sensitivity found in the present study might be the collection of 3 ml of whole blood for DNA extraction in place of 1 ml and a slight modification of the recommended DNA extraction protocol (13). The collection of 3 ml of whole blood ensured the presence of at least 1 bacterium in the sample, based on the observation that in children below 15 years of age, the level of bacteremia ranged from <0.3 to 387 CFU/ml, with a median of 1 CFU of *S. enterica* serovar Typhi/ml of blood, of which a mean of 63% were intracellular (14). The negative predictive value (PV^-) of PCR was found to be better than that of blood culture. As some of the cases that tested positive exclusively by the Widal test may not be true cases of typhoid, the observed PV^- (60%) in this study may be less than the actual rate of positivity. For assigning a test to be of clinical utility, it is recommended that the LR^+ and LR^- of the test should be ≥10 and ≤0.1, respectively (5). In the present study, the LR^+ and LR^- of PCR was found to be ∞ and 0.07, respectively.

Considering PCR as the gold standard, the most appropriate diagnostic cutoff titer of TO and TH have been evaluated at the two different cutoff titers, i.e., 80 for O agglutinin, 160 for H agglutinin, and 160 for O and/or H agglutinins. It was found that specificity was significantly higher with the latter titer (Table 1).

It is important to note that a total of 11 cases were negative by Widal at this titer but were positive exclusively by PCR, with a mean day of presentation of 5.3 days. There were five other cases which were PCR and culture positive, with a mean day of presentation of 3.7 days (Table 2). These observations suggest that the nested PCR assay can be used in the early diagnosis of typhoid fever, which will not only reduce morbidity, mortality, and acquisition of the carrier state but will also reduce the transmission of the disease. Further, it was observed that the Widal test seems to be relevant in the second week of illness at the proposed titer, as it failed to detect 28% of the total typhoid cases that presented in the first week of illness.

TABLE 2. Status of PCR, blood culture, and Widal test and mean duration of illness in suspected cases of typhoid fever

No. of cases (%)	Nested PCR	Blood culture	Widal test	Mean duration of illness (days)
12 (21)	+	+	+	9.5
25 (44)	+	−	+	9.1
4 (7)	−	−	+	7.5
11 (19)	+	−	−	5.3
5 (9)	+	+	−	3.7
All cases 57 (100)	53	17	41	

It may, therefore, be concluded that nested PCR must be considered the gold standard test in the diagnosis of typhoid fever in order to determine the biostatistical parameters of Widal and other simpler serological tests to be used in field conditions.

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